

# Inhibition of Hepatic Metastases of Human Colon Cancer in Nude Mice by a Chimeric SF-25 Monoclonal Antibody

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**Background/Aims:** Hepatic metastasis is one of the most serious complications of human colon cancer. A murine-human chimeric SF-25 monoclonal antibody was prepared, and this construct recognizes a cell surface antigen highly present in human colon adenocarcinoma. **Methods:** This study determined if the chimeric SF-25 monoclonal antibody inhibits the outgrowth of hepatic metastases of human colon adenocarcinoma using an athymic nude mouse model. **Results:** A single intravenous injection of chimeric SF-25 monoclonal antibody significantly inhibited the outgrowth of 5- and 7-day hepatic micrometastases ( $P = 0.0001$  and  $0.004$ , respectively, vs. untreated) and improved the survival of the animals. No detectable tumor was found in the liver when mice were treated by multiple injections of the antibody immediately after tumor cell grafting into the portal vein. In contrast,  $F(ab')_2$  fragments did not show antitumor effects, and the administration of natural killer cell or macrophage depleting agents (anti-asialo GM1 antibody and carrageenan, respectively) substantially inhibited the antitumor effects of chimeric SF-25 monoclonal antibody in vivo. **Conclusions:** Chimeric SF-25 monoclonal antibody inhibits growth of hepatic metastasis of human colon cancer, and cell-mediated host immune mechanisms seem to be important for its in vivo antitumor activity.

Despite major advances in general patient care and surgical therapy, the mortality rate associated with colon adenocarcinoma has not changed significantly during the last 40 years, principally because of metastatic spread and tumor recurrence. Although 70%–80% of patients will present with operable primary tumors at the time of diagnosis, surgery alone is often unable to permit a long-term survival even after complete surgical resection because of metachronous metastatic spread and tumor recurrence. The liver is the primary site of metastasis in patients with colon cancer.<sup>1</sup> At autopsy, liver involvement is frequently found in patients with colon cancer and is the sole site of metastasis in one third of patients.<sup>2</sup> Those patients who develop hepatic involvement, either solitary or multiple, are incurable in the vast majority of cases, and it is generally accepted that

the presence of hepatic metastases is associated with a poor prognosis.

Metachronous hepatic metastases arise from the tumor cells that are seeded into the portal vein system from the primary site or micrometastases that are already present in the liver at the time of surgery. The prevention of these hepatic metastases has been the subject of great interest in the treatment of human colon cancer. In this regard, inhibition of tumor cell spread via the vascular system as well as the treatment of synchronous hepatic micrometastases are important. Numerous trials of post-operative adjuvant treatment regimens have been performed in an attempt to protect from metastatic spread and tumor recurrence, but most have not shown a reduction in the incidence of hepatic metastasis.<sup>3–6</sup> The death rate will undoubtedly remain the same until improved methods for treatment of hepatic involvement become available. Thus, it is of general interest to develop and explore new experimental approaches that may eventually be translated into useful clinical treatment regimens for this disease.

A murine SF-25 monoclonal antibody (MAb) has been found to recognize a 125-kilodalton cell surface antigen highly expressed in transformed human cells.<sup>7</sup> Metabolic and cell surface labeling studies have shown that SF-25 antigen was a disulfide bond-linked heterodimer composed of 35-kilodalton and 90-kilodalton glycosylated subunits termed  $\alpha$  and  $\beta$ , and the epitope was located on the core protein of  $\beta$  subunit (unpublished data, January 1992). The SF-25 antigen was expressed in all cases of surgically obtained human colon adenocarcinoma, and the distribution of antigen in the tissues was uniform. A number of normal human tissues were found to be negative by immunohistological staining, including the stomach, colon, small intestine, liver, lung, adrenal gland, and other tissues.<sup>7,8</sup> Exceptions were the kidney

**Abbreviations used in this paper:** ADCC, antibody-dependent cell-mediated cytotoxicity; CGN, carrageenan; MAb, monoclonal antibody; NK, natural killer.

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and pancreas. Positive staining was present in a subpopulation of proximal tubular cells of kidney, and very weak staining was also observed in islet cells of the pancreas. Furthermore, SF-25 antibody was shown to be immunolocalized by nuclear imaging studies to hepatic metastasis of human colon adenocarcinoma established in athymic nude mice.<sup>8</sup>

It is well recognized that repeated injections of murine MAb result in the production of human anti-mouse antibody in patients with colorectal carcinoma.<sup>9,10</sup> The human anti-mouse antibody binds to murine MAb and accelerates clearance from the circulation. In addition, the anti-mouse immune response causes some degree of toxicity and may be responsible for allergic reaction.<sup>9</sup> To potentially achieve a more clinically useful reagent, we have investigated a chimeric SF-25 MAb in which the constant regions of human immunoglobulin G1 were linked to the variable regions of murine SF-25 MAb by recombinant DNA techniques. This chimeric antibody has been shown to be useful for targeting of human effector cells to the human colon adenocarcinomas established in the liver of severe combined immunodeficient mice.<sup>11</sup> In the present investigation, we have studied the properties of this mouse-human chimeric SF-25 MAb and examined if unmodified chimeric antibody has an inherent antitumor activity and prevents the outgrowth of hepatic metastases of human colon cancer in an experimental metastasis model established in nude mice. We report here that chimeric SF-25 MAb was able to inhibit the growth of human colon cancer cells that are injected into the portal vein as well as micrometastases established in the liver of the animals.

## Materials and Methods

### Cell Lines

All cell lines were maintained in Dulbecco's modified Eagle medium (M.A. Bioproducts, Walkersville, MD) supplemented with 10% fetal calf serum inactivated at 56°C for 30 minutes, 10  $\mu$ mol/L nonessential amino acids, 100 U/mL penicillin, and 100  $\mu$ g/mL streptomycin. In some experiments, cells were maintained in RPMI 1640 (Hazleton Biologics, Inc., St. Lenexa, KS) supplemented with 10% heat-inactivated fetal calf serum, 10  $\mu$ mol/L nonessential amino acids, 2 mmol/L L-glutamine, 100 U/mL penicillin, and 100  $\mu$ g/mL streptomycin.

Cells used for in vitro testing were harvested from the monolayer cultures by treatment with 0.04% ethylenediaminetetraacetic acid (EDTA) solution in Hank's balanced salts in the absence of trypsin for 5 minutes at 37°C because SF-25 antigen was sensitive to trypsin treatment. All of the cell lines used were shown to be free of mycoplasma contamination by a nucleic acid hybridization technique using Mycoplasma T.C. 11 (Gen-Probe Inc., San Diego, CA).

### Production of the Chimeric MAB

A chimeric SF-25 MAb has been produced by the same methods as previously reported.<sup>12,13</sup> In brief, human-mouse chimeric immunoglobulin genes were constructed by joining L and H chain variable region genes isolated from SF-25 MAb-secreting hybridoma cells to human  $\kappa$  and  $\gamma$  1 constant region genes, respectively. These constructs were then transfected into Sp2/0 myeloma cells to produce the chimeric MAB.

### Purification and Radiolabeling of Antibodies

Murine SF-25 MAb (isotype, murine immunoglobulin G1) and chimeric SF-25 MAb (isotype, human immunoglobulin G1) were purified by Sepharose CL-4B Staphylococcal Protein A-affinity column (Pharmacia LKB Biotechnology Inc., Piscataway, NJ). Radiolabeling with <sup>125</sup>I was performed by the Iodogen method.<sup>14</sup> Radiolabeled MAB was separated from free <sup>125</sup>I by Sephadex G-25 column. Specific activities of iodinated MABs were calculated as a ratio of counts per minute of radioisotope attached per mole of antibody.

### Association Constant

A constant number of LS 180 cells ( $10^5$ /well) was incubated with 100  $\mu$ L of <sup>125</sup>I-labeled SF-25 MAb serially diluted with phosphate-buffered saline (PBS) containing 20% bovine serum for 4 hours at 4°C using 96-well filter-bottomed plates (V & P Scientific Inc., San Diego, CA). Cells were then washed five times with PBS to remove unbound antibody, and bound radioactivity was counted in the gamma well counter. Concentration of MABs and the amount of bound MABs were calculated from radioactivity (cpm) and specific activity (cpm/mol) of <sup>125</sup>I-labeled MABs. The association constant of MAB and the number of antibody binding sites per tumor cell were determined by the methods and equation described by Frankel and Gerhard.<sup>15</sup>

### Flow Cytometric Analysis of SF-25 MAB Binding to LS 180 Tumor Cell Surface

One million LS 180 cells were incubated for 1 hour at 4°C with 200  $\mu$ L of murine SF-25 or B3/25 MAB (Boehringer Mannheim, Indianapolis, IN) adjusted to 10  $\mu$ g/mL in 0.01 mol/L PBS (pH 7.2) containing 1% bovine serum albumin. B3/25 MAB binds to human transferrin receptor, which internalizes rapidly at 37°C.<sup>16</sup> After washing three times with cold PBS, the cells were incubated at 4°C or 37°C for 15 minutes to 1 hour and then reacted for 1 hour at 4°C with 200  $\mu$ L of fluorescein isothiocyanate-conjugated goat anti-mouse immunoglobulin G (Cappel, Cochranville, PA) diluted 1:100 in PBS containing 1% bovine serum albumin. After washing three times with cold PBS and fixed with 2% paraformaldehyde, fluorescence was detected in a Becton Dickinson FACScan (Becton Dickinson, Mountain View, CA). Results are expressed as mean percentage of positive staining cells.

### Complement-Mediated Cytotoxicity Assay

LS 180 cells were used as target cells. Confluent LS 180 cells were harvested with 0.04% EDTA solution and radiolabeled by incubating  $1 \times 10^6$  cells with 100  $\mu\text{Ci}$  of  $^{51}\text{Cr}$  (New England Nuclear, Boston, MA) for 30 minutes at  $37^\circ\text{C}$ . The radiolabeled cells were washed with PBS and adjusted to  $1 \times 10^5$  mL in RPMI 1640. Guinea pig, mouse, and human complement serum (Sigma Chemical Co., St. Louis, MO) were absorbed before use by incubating 1 mL of each serum with  $10^7$  LS 180 cells for 30 minutes at  $4^\circ\text{C}$  and centrifuged. One hundred microliters of target cells and 50  $\mu\text{L}$  of chimeric SF-25 MAb (1–1000  $\mu\text{g}/\text{mL}$  in RPMI 1640) were pipetted into 96-well U-bottom plates, and then 100  $\mu\text{L}$  of 1:5 dilution of complement serum was added to each well. Plates were incubated in a  $\text{CO}_2$  incubator at  $37^\circ\text{C}$  for 30 minutes. After centrifugation of the U-bottom plates at 1500 rpm for 15 minutes, 200  $\mu\text{L}$  of culture supernatant was collected and radioactivity was determined by a gamma well counter. As a positive control, we used anti-LS 180 polyclonal antibody (a gift from Dr. Sabro Sone, Toray Industries, Tokyo, Japan) that was prepared by immunization of rabbit with LS 180 cells and Freund's complete adjuvant. Spontaneous release of  $^{51}\text{Cr}$  was measured after incubation of target cells alone with RPMI 1640, and total count was determined after incubation of cells in 1.0 N HCl. Spontaneous release was  $<10\%$  of total release in all experiments. The percent specific lysis was determined by the following formula:

$$\frac{\text{Observed cpm} - \text{Spontaneous cpm}}{\text{Total cpm} - \text{Spontaneous cpm}} \times 100 = \% \text{ Specific Lysis.}$$

### Antibody-Dependent Cell-Mediated Cytotoxicity Assay

An antibody-dependent cell-mediated cytotoxicity (ADCC) assay was performed using  $^{51}\text{Cr}$ -labeled LS 180 cells as the target cells. One hundred microliters of target cells adjusted to  $2 \times 10^5/\text{mL}$  of RPMI 1640 and 50  $\mu\text{L}$  of MAb (100  $\mu\text{g}/\text{mL}$  in RPMI 1640) were pipetted into 96-well U-bottom plates, and then 100  $\mu\text{L}$  of various concentrations of murine effector cells prepared according to the methods described below were added to each well. Final concentration of MAb in ADCC was adjusted to 20  $\mu\text{g}/\text{mL}$  because this concentration was optimal as shown by preliminary experiments. After plates were incubated in a  $\text{CO}_2$  incubator at  $37^\circ\text{C}$  for 8 hours, radioactivity of supernatant was determined by a gamma well counter and the percent specific lysis was determined as described above.

### Animals

Four to five-week-old female athymic Balb/c nude mice were obtained from the Harlan Sprague-Dawley Inc. (Indianapolis, IN). Throughout the experiments, these animals were maintained under specific pathogen-free conditions. All animal experiments were approved by the Committee on Research

Animal Care protocol review group and performed according to Massachusetts General Hospital guidelines.

### Preparation of Thioglycollate-Elicited Macrophages

Nude mice were inoculated intraperitoneally with 1 mL of thioglycollate medium (Difco) (VWR Scientific Corp., Philadelphia, PA). Four days later, thioglycollate-elicited macrophages were collected from the peritoneal cavity. The purity of macrophages was  $>90\%$  when examined by flow cytometry using F4/80 MAb, a rat MAb specific to murine macrophages.<sup>17</sup>

### Preparation of Murine Splenocytes and Isolation of Natural Killer Cells

Mice were anesthetized with ether and killed by axillary bleeding. The peritoneal cavity was exposed under sterile conditions, and the spleen was excised into a plastic dish containing 5 mL of RPMI 1640 medium. Next, a small incision was made in the spleen, and splenocytes were isolated by squeezing the spleen repeatedly with forceps. Splenocytes were then transferred to a plastic tube and washed twice with RPMI 1640 medium. Adherent cells were removed from murine splenocytes after incubation in a plastic dish for 2 hours at  $37^\circ\text{C}$ , and murine natural killer (NK) cells were isolated by discontinuous density gradient centrifugation.<sup>18,19</sup> Nonadherent splenocytes were adjusted to  $5 \times 10^7$  cells/gradient and separated into fractions by centrifugation at 300g for 1 hour at  $20^\circ\text{C}$  on a discontinuous density gradient of Percoll at concentrations of 20%, 40%, 50%, 60%, 70%, and 100% (osmotic pressure adjusted at 320 mOsm/kg using  $10 \times$  PBS). Large granular lymphocytes were collected from the interphases between 20% and 40% and between 40% and 50% concentrations of Percoll. The purity of murine NK cells was examined by flow cytometric analysis using rabbit anti-serum to ganglio-N-tetraosylceramide (anti-asialo GM1) (Wako Chemicals, Dallas, TX)<sup>20</sup> and was more than 90%.

### Hepatic Metastasis of Human Colon Cancer Established in Athymic Nude Mice

Hepatic metastasis of human colon cancer was established by the methods described by Kozlowski et al. with some modification.<sup>21</sup> A human colonic carcinoma cell line, LS 180, was harvested from tissue culture by overlaying subconfluent monolayer cultures with 0.05% trypsin and 0.02% EDTA solution in Hank's balanced salt solution (Gibco Laboratories, Grand Island, NY) for 5 minutes and washed three times with PBS. In some experiments, when treatment started immediately after the tumor cell injection, cells were harvested with 0.04% EDTA solution as described above. Nude mice were anesthetized with 0.4 mL of 2% chloral hydrate (Sigma Chemical Co.) by intraperitoneal injection and placed in the decubitus position. A transverse incision was made in the left flank through the skin and peritoneum, exposing the spleen. Mice were injected with  $1.0 \times 10^6$  LS 180 cells in 0.25 mL PBS into the portal vein through splenic hilus using a 27-gauge

needle. After waiting a few minutes, when hemostasis of spleen was ascertained, the abdominal cavity was closed. Visible tumor foci are generally formed in the liver within 5 days after tumor cell injection. They grew to a few millimeters in diameter by day 7.

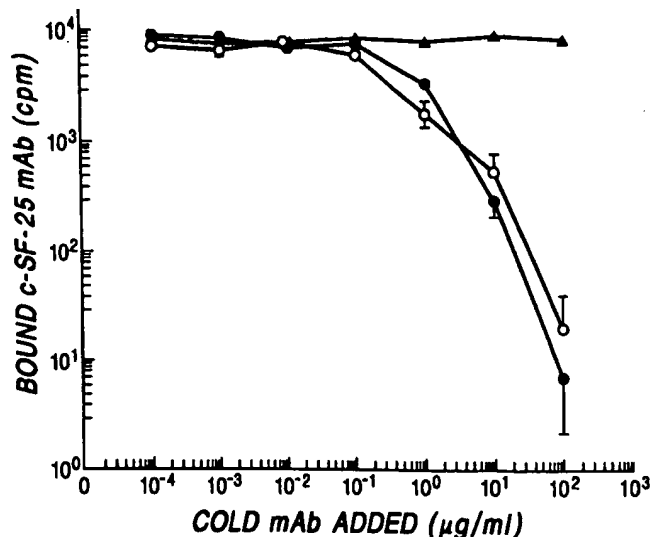
### In Vivo Effect of Chimeric SF-25 MAb

The *in vivo* antitumor effect of chimeric SF-25 MAb was studied using the above described hepatic metastatic model. Mice were intravenously injected into the tail vein with 100  $\mu$ L of PBS (untreated control group), chimeric SF-25 MAb, or F(ab')<sub>2</sub> fragment of chimeric SF-25 MAb at the dose as indicated in Results. Levels of endotoxin concentration in MABs used in our experiments were tested by Limulus Amebocyte Lysate test (QCL-1000 Chromogenic LAL; Bio-Whittaker, Inc., Walkersville, MD) and were always <0.2 EU per mg of MAB. If necessary, we removed endotoxin from MABs using END-X B15 endotoxin removal kit (Association of Cape Cod, Inc., Woods Hole, MA). We killed mice 5 weeks after the tumor cell injection when untreated control animals begin to die because of the large tumor mass. The liver was fixed in formaldehyde, and the weight of all hepatic tumors was determined after careful separation from the normal surrounding liver. The survival rate of mice treated with chimeric SF-25 MAb was also examined and compared with the life span of untreated animals.

### In Vivo Depletion of Murine Macrophage and NK Cells

Antitumor effect of chimeric SF-25 MAb after *in vivo* depletion of murine macrophages and NK cells was examined using animals bearing 5-day hepatic micrometastases. Because  $\lambda$ -carrageenan (CGN) (Sigma Chemical Co.) is toxic to macrophages,<sup>22,23</sup> this reagent was chosen as a macrophage-depleting agent. However, native  $\lambda$ -CGN is not water soluble at low temperature, and the sulfate groups contained in the  $\lambda$ -CGN have been shown to produce mitogenic effects.<sup>23</sup> To prepare a water-soluble reagent and to avoid nonspecific stimulation of the immune system *in vivo*,  $\lambda$ -CGN was desulfated according to the method of Ishizaka et al.<sup>22</sup> After tumor cell injection, mice were injected intraperitoneally with 3 mg of this desulfated CGN dissolved in PBS every other day for 5 days (total of 9 mg/mouse) to deplete macrophages and then treated with chimeric SF-25 MAb. To confirm that macrophages were depleted, flow cytometric analysis was performed using anti-macrophage MAb (F4/80). It was found that macrophages in peripheral blood decreased from 18.0% to 1.9% by intraperitoneal injection of desulfated CGN.

For NK cell depletion *in vivo*, nude mice were injected intravenously with 20  $\mu$ g of anti-asialo GM1 rabbit serum 5, 7, 9, and 12 days after tumor cell injection, and the antitumor effect of chimeric SF-25 MAb was tested. The percentage of NK cells in peripheral blood reduced from 7% to an undetectable level after NK cell depletion



**Figure 1.** Competitive inhibition of <sup>125</sup>I-labeled chimeric SF-25 MAb binding to LS 180 cells by unlabeled chimeric SF-25 and murine SF-25 MABs. The binding of <sup>125</sup>I-labeled chimeric SF-25 MAB was inhibited by both chimeric SF-25 and murine SF-25 MABs. In contrast, this binding was not inhibited by nonrelevant MAB (B2TT: antitetanus toxoid MAB). These results show that the chimeric SF-25 MAB recognizes the same epitope as the murine MAB. ○, chimeric SF-25 MAB; ●, murine SF-25 MAB; ▲, nonspecific MAB (B2TT).

when examined by flow cytometric analysis using anti-asialo GM1 serum.

### Expression of SF-25 Antigen in LS 180 Tumor Cells After Treatment With Chimeric SF-25 MAB

To examine the expression of SF-25 antigen in LS 180 tumor cells after chimeric SF-25 MAB treatment, a single cell suspension of LS 180 cells was prepared from hepatic tumors derived from chimeric SF-25 MAB-treated and -untreated mice, and the expression of SF-25 antigen was examined by flow cytometry using fluorescein isothiocyanate-labeled SF-25 MAB.

### Statistical Analysis

The differences in tumor weights were tested by Kruskal-Wallis test followed by Mann-Whitney rank sum test with Bonferroni correction to conduct multiple comparison.<sup>24</sup> The survival rate was compared with the untreated control group using the Mantel-Haenszel test.<sup>25</sup>

## Results

### Properties of Chimeric SF-25 MAB

The chimeric SF-25 and murine SF-25 MABs showed identical physical properties with respect to its immunoreactivity and association constant. Figure 1 shows a competitive inhibition assay using <sup>125</sup>I-labeled chimeric SF-25 MAB. The binding of <sup>125</sup>I-labeled chimeric SF-25 MAB to the LS 180 cell line was completely

**Table 1.** Flow Cytometric Analysis of SF-25 MAb Binding to LS 180 Cells

	Percentage of positive staining cells (min) (%)			
	0	15	30	60
SF-25 MAb				
4°C	98.4	97.2	97.7	96.5
37°C	98.4	97.0	89.7	85.6
B3/25 MAb				
4°C	49.8	42.9	43.8	49.0
37°C	49.8	28.0	20.8	18.7

NOTE. Because SF-25 antigen did not internalize or shed, the binding of SF-25 MAb to the tumor cell surface was stable at both 4°C and 37°C. By contrast, percentage of B3/25 MAb-positive cells decreased at 37°C because of the internalization of transferrin receptor.

inhibited by both cold murine SF-25 and chimeric SF-25 but not by B<sub>2</sub>TT, a nonspecific MAb. Thus, the chimeric SF-25 and murine SF-25 MAbs recognize the same or structurally very close epitopes. The association constant of chimeric SF-25 and murine SF-25 MAbs were calculated to be  $2.4 \times 10^9$  L/mol and  $1.9 \times 10^9$  L/mol, respectively. These binding constants indicate that the antigen-antibody interaction by both chimeric SF-25 and murine SF-25 MAbs is of high avidity. The number of antibody binding sites per cell (LS 180) was approximately  $2.5 \times 10^5$  for both the chimeric and original murine MAbs. The binding of MAbs to the tumor cell surface after incubation at 4°C or 37°C was examined using flow cytometry (Table 1). It was found that the number of B3/25 MAb-positive cells rapidly decreased from 49.8% to 18.7% after 60 minutes of incubation at 37°C because bound B3/25 MAb was internalized along with the transferrin receptor at this temperature. In contrast, SF-25 MAb-positive cells did not change significantly at both 4°C and 37°C. This data indicates that SF-25 MAb is not internalized or shed from the tumor cell surface on binding to the antigen. Similar results were obtained when <sup>125</sup>I-labeled SF-25 MAbs were used to examine the amount of cell-bound, free, and internalized antibodies after various incubation times at 37°C (unpublished data, October 1990).

#### Complement-Mediated Cytotoxicity Against LS 180 Cells

Figure 2 shows the percent specific lysis of LS 180 cells by complement-mediated cytotoxicity using guinea pig serum as a complement source. Rabbit anti-LS 180 polyclonal antibody produced substantial cytotoxicity against LS 180 in the presence of guinea pig complement, but cytotoxicity was not elicited by chimeric SF-25 MAb under the same experimental conditions. Similarly, complement-mediated cytotoxicity was not produced by

chimeric SF-25 MAb using mouse or human complement (data not shown).

#### ADCC Produced by Murine NK Cells and Macrophages

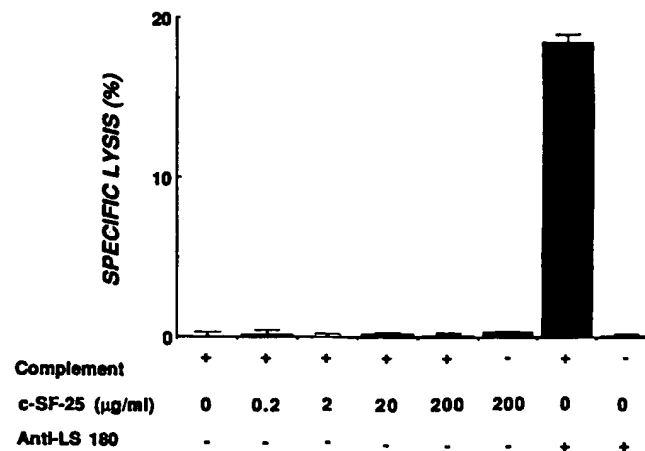
ADCC activities produced by murine NK cells and macrophages in the presence of chimeric SF-25 MAb were examined by a <sup>51</sup>Cr release assay. Figure 3 shows the results of an 8-hour cytotoxicity assay because a 4-hour assay did not produce substantial cytotoxicity. Figure 3A shows the ADCC by murine NK cells isolated from splenocytes by discontinuous density gradient centrifugation. The purified murine NK cells showed ADCC in the presence of chimeric SF-25 MAb. Figure 3B shows the results obtained with thioglycollate-elicited murine macrophages and shows that these cells mediated ADCC at various effector to target ratios.

#### Animal Model of Hepatic Metastatic Disease

Hepatic metastases of human colon adenocarcinoma were established by injecting LS 180 cells into the portal vein of nude mice. Figure 4 shows a representative example of hepatic metastases established in nude mice 5 weeks after injection of LS 180 cells.

#### Inhibition of Tumor Cells Grafted Into Portal Vein

It was first determined if chimeric SF-25 MAb would inhibit the growth of tumor cells that have been seeded into the portal vein. Chimeric SF-25 MAb (1.0, 10, and 100 µg/mouse) was intravenously injected into mice for 4 consecutive days (immediately after and 1, 2,



**Figure 2.** Cytotoxicity of LS 180 cells by complement-mediated cytotoxicity using guinea pig complement. Cytotoxicity was elicited by rabbit anti-LS 180 polyclonal antibody but not by chimeric SF-25 MAb in the presence of guinea pig complement.

and 3 days after tumor cell grafting into the portal vein). Five weeks later, the weight of hepatic metastatic tumors from each mouse was measured. Results are shown in Figure 5A. Tumor weight of mice injected with PBS was  $1.11 \pm 1.51$  g (mean  $\pm$  SD;  $n = 10$ ). The chimeric SF-25 MAb treatment significantly inhibited the tumor growth (Kruskal–Wallis test;  $P = 0.0005$ ). In particular, mice injected with 10 or 100  $\mu$ g ( $n = 10$ , respectively) were free of detectable tumor in the liver at the time of killing (1.0  $\mu$ g/mouse;  $0.37 \pm 0.83$  g;  $n = 10$ ).

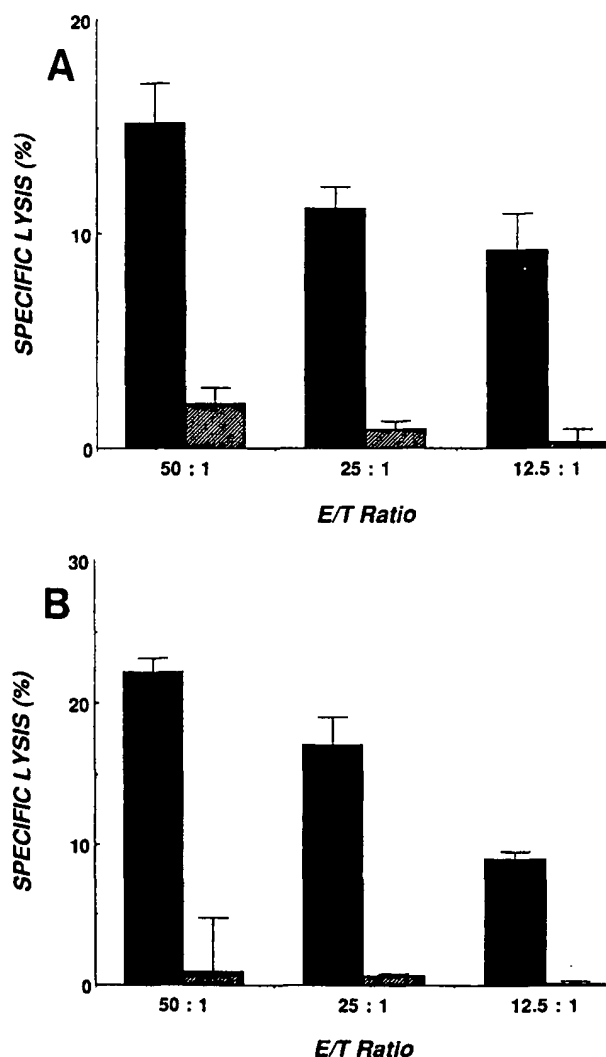
### Inhibitions of 5- and 7-Day Hepatic Metastases

The treatment was initiated 5 days after tumor cell injection to test if chimeric SF-25 MAb inhibits the

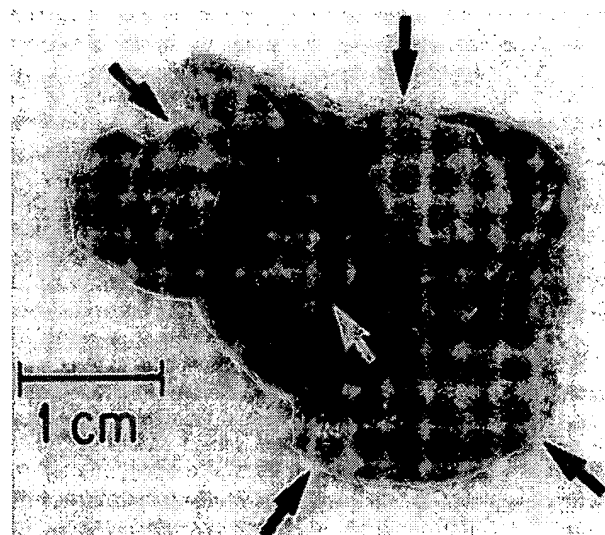
outgrowth of tumors derived from hepatic micrometastases already present at the time of treatment. A single intravenous injection of chimeric SF-25 MABs (100  $\mu$ g/mouse) produced a substantial antitumor effect on 5-day hepatic tumors (Figure 5B). Nine of 10 mice were found to be free of detectable hepatic tumors at the time of killing, and only 1 mouse developed a hepatic tumor of  $<1$  mg. The antitumor effect of chimeric SF-25 MAB was statistically significant by Mann–Whitney rank sum test with Bonferroni correction ( $P = 0.0001$  vs. untreated;  $1.14 \pm 1.30$  g;  $n = 18$ ). In contrast, the  $F(ab')_2$  fragment of chimeric SF-25 MAB was less effective, and statistically significant antitumor effect was not observed ( $1.20 \pm 1.77$  g;  $n = 10$ ;  $P = 0.3228$  vs. untreated group). Mice were also treated with various doses of chimeric SF-25 MAB (0.1, 1.0, or 10  $\mu$ g per mouse) 7 days after tumor cell injection when there were larger tumor burdens in the liver. Antitumor effect was observed in a dose-dependent manner (untreated,  $1.87 \pm 1.80$  g,  $n = 15$ ; 0.1  $\mu$ g of chimeric SF-25 MAB/mouse,  $1.37 \pm 1.67$  g,  $n = 9$ ; 1.0  $\mu$ g of chimeric SF-25 MAB/mouse,  $0.53 \pm 0.70$  g,  $n = 8$ ; 10  $\mu$ g of chimeric SF-25 MAB/mouse,  $0.07 \pm 0.09$  g,  $n = 7$ ) ( $P = 0.025$  by Kruskal–Wallis test), and statistically significant antitumor effect was observed at 10  $\mu$ g per mouse ( $P = 0.025$  by Mann–Whitney rank sum test) (Figure 5B).

### Effect of Chimeric SF-25 MAB on Animal Survival

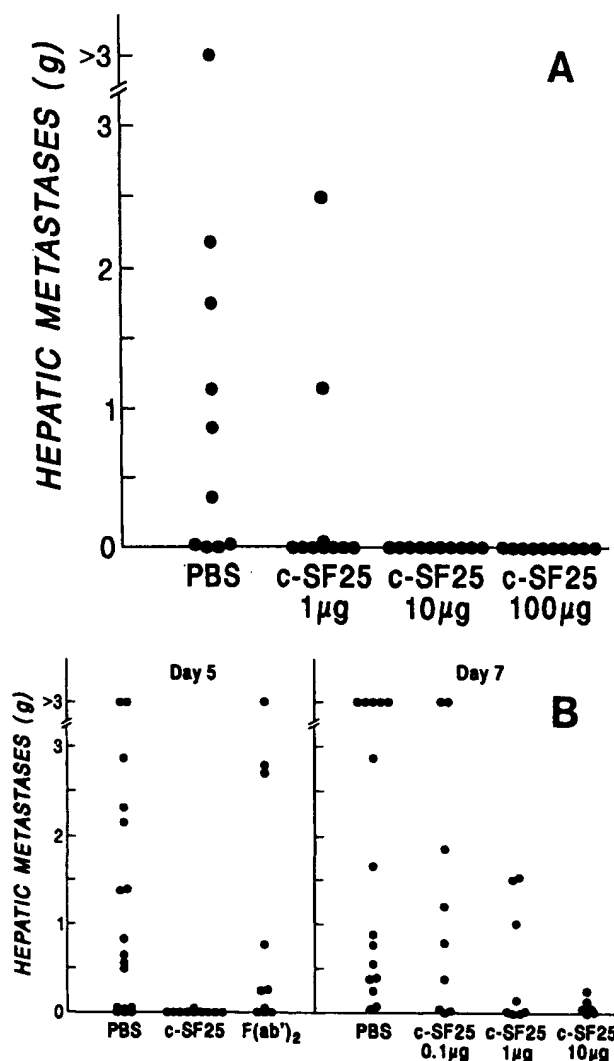
The length of survival of nude mice injected with chimeric SF-25 MAB (100  $\mu$ g per mouse) 5 days after



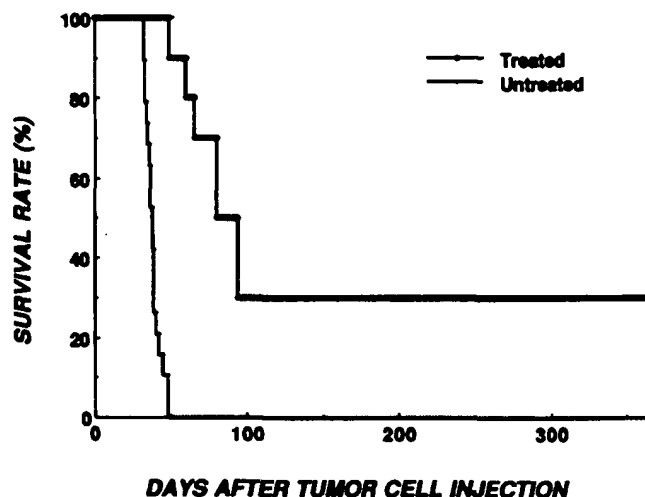
**Figure 3.** ADCC by murine effector cells in an 8-hour  $^{51}\text{Cr}$  release assay. (A) Murine NK cells purified from splenocytes showed ADCC activity against LS 180 cells in the presence of chimeric SF-25 MAB. (B) Thioglycollate-elicited murine macrophages also elicited ADCC against LS 180 cells in the presence of chimeric SF-25 MAB. ■, chimeric SF-25 MAB; ▨, MAB.



**Figure 4.** Hepatic metastases of human colon adenocarcinoma established in nude mice. The majority of mice injected with LS 180 cells developed large "cannon balllike" metastasis in the liver as indicated by arrows.



**Figure 5.** Effect of chimeric SF-25 MAb on hepatic metastases. Tumors were carefully separated from surrounding normal liver after formaldehyde fixation and weighed in a balance. Vertical axis denotes the weight of hepatic metastases. The differences in tumor weights were tested by Kruskal-Wallis test followed by Mann-Whitney rank sum test with Bonferroni correction. (A) Inhibition of tumor cells grafted into portal vein. The effect of different treatments (Kruskal-Wallis test;  $P = 0.0005$ ) is given as follows: untreated mice vs. mice treated with 1 µg chimeric SF-25 MAb, 10 µg chimeric SF-25 MAb, or 100 µg chimeric SF-25 MAb ( $P = 0.0344$ ,  $P = 0.0003$ , and  $P = 0.0003$ , respectively). Statistically significant  $\alpha$  level is  $0.05/3 = 0.0167$ . (B) Inhibition of 5-day and 7-day hepatic micrometastases. The effect of different treatments on 5-day hepatic micrometastases (Kruskal-Wallis test;  $P = 0.005$ ) is given as follows: untreated mice vs. mice treated with 100 µg chimeric SF-25 MAb or 100 µg F(ab')<sub>2</sub> fragment of chimeric SF-25 MAb ( $P = 0.0001$  and  $P = 0.3228$ , respectively); mice treated with 100 µg chimeric SF-25 MAb vs. mice treated with 100 µg F(ab')<sub>2</sub> fragment of chimeric SF-25 MAb ( $P = 0.0021$ ). Statistically significant  $\alpha$  level is  $0.05/3 = 0.0167$ . The effect of different treatments on 7-day hepatic micrometastases (Kruskal-Wallis test;  $P = 0.025$ ) is given as follows: untreated mice vs. mice treated with 0.1 µg chimeric SF-25 MAb, 1 µg chimeric SF-25 MAb, or 10 µg chimeric SF-25 MAb ( $P = 0.1949$ ,  $P = 0.0192$ , and  $P = 0.0009$ , respectively). Statistically significant  $\alpha$  level is  $0.05/3 = 0.0167$ .

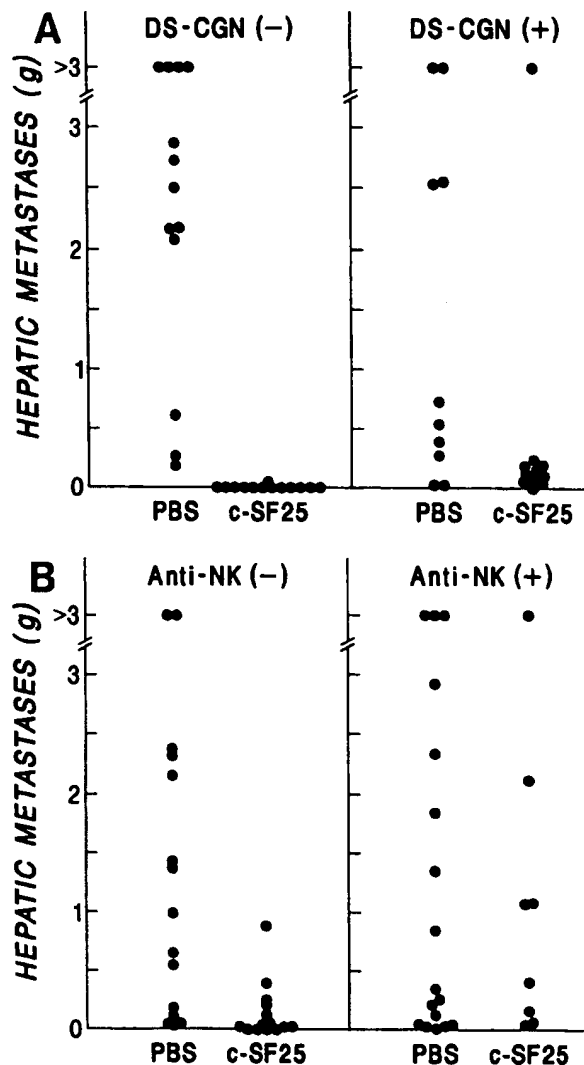


**Figure 6.** Kaplan-Meier curves of mice treated with chimeric SF-25 MAb. All mice treated with chimeric SF-25 MAb survived more than 6 weeks, whereas all untreated animals died within 6 weeks after LS 180 cell injection. The survival rate of treated animals was significantly longer than untreated controls ( $P < 0.000001$  vs. untreated by Mantel-Haenszel test).

the tumor cell injection was determined. Figure 6 shows the survival curve of mice treated with chimeric SF-25 compared with untreated animals. It is noteworthy that all untreated mice died within 6 weeks after tumor cell injection due to massive hepatic metastasis (survival, 32–48 days; median survival, 38 days;  $n = 19$ ). In contrast, all animals treated with chimeric SF-25 MAb survived longer than untreated mice and 3 of 10 animals were free of hepatic tumor when removed from the study 1 year after tumor cell injection (survival, 49–>365 days; median survival, 80 days;  $n = 10$ ). The improved survival rate of treated animals was highly significant ( $P < 0.000001$  vs. untreated by Mantel-Haenszel test).

#### Antitumor Effect of Chimeric SF-25 MAb After Macrophage and NK Cell Depletion

Because F(ab')<sub>2</sub> of chimeric SF-25 MAb was less effective than the whole chimeric SF-25 MAb, the Fc fragment of the antibody seemed to be essential for its antitumor effect, and such observed effects may be cell mediated. Macrophages and NK cells are major effector cells that express immunoglobulin G Fc receptors in nude mice. Therefore, we examined if antitumor activity of chimeric SF-25 MAb is inhibited by macrophage and NK cell depletion. Mice were injected intraperitoneally with desulfated CGN to deplete macrophages after the injection of LS 180 cells, and then the antitumor effects of chimeric SF-25 MAb (100 µg per mouse 5 days after tumor cell injection) were retested under these conditions. The results are shown in Figure 7A. The injection of desulfated CGN did not affect the growth of hepatic



**Figure 7.** Antitumor effect of chimeric SF-25 MAb after in vivo depletion of murine macrophages and NK cells. (A) Macrophage depletion by desulfated CGN. The effect of different treatments (Kruskal-Wallis test;  $P = 0.0001$ ) is given as follows: untreated mice vs. mice treated with 100  $\mu$ g chimeric SF-25 MAb alone, desulfated CGN alone, or desulfated CGN and 100  $\mu$ g chimeric SF-25 MAb ( $P = 0.00003$ ,  $P = 0.1561$ , and  $P = 0.00034$ , respectively); mice treated with 100  $\mu$ g chimeric SF-25 MAb alone vs. mice treated with desulfated CGN alone or desulfated CGN and 100  $\mu$ g chimeric SF-25 MAb ( $P = 0.00003$  and  $P = 0.00005$ , respectively); mice treated with desulfated CGN alone vs. mice treated with desulfated CGN and 100  $\mu$ g chimeric SF-25 MAb ( $P = 0.0244$ ). Statistically significant  $\alpha$  level is  $0.05/6 = 0.0083$ . (B) NK cell depletion by anti-asialo GM1 antibody. The effect of different treatments (Kruskal-Wallis test;  $P = 0.005$ ) is given as follows: untreated mice vs. mice treated with 100  $\mu$ g chimeric SF-25 MAb alone, anti-NK alone, or anti-NK and 100  $\mu$ g chimeric SF-25 MAb ( $P = 0.0006$ ,  $P = 0.3745$ , and  $P = 0.3485$ , respectively); mice treated with 100  $\mu$ g chimeric SF-25 MAb alone vs. mice treated with anti-NK alone or anti-NK and 100  $\mu$ g chimeric SF-25 MAb ( $P = 0.0023$  and  $P = 0.0023$ , respectively); mice treated with anti-NK alone vs. mice treated with anti-NK and 100  $\mu$ g chimeric SF-25 MAb ( $P = 0.4286$ ). Statistically significant  $\alpha$  level is  $0.05/6 = 0.0083$ .

metastasis (untreated mice,  $2.61 \pm 1.69$  g,  $n = 13$ ; mice treated with desulfated CGN,  $1.50 \pm 1.61$  g,  $n = 10$ ;  $P = 0.1561$ ). A single intravenous injection of chimeric SF-25 MABs (100  $\mu$ g per mouse) produced a substantial antitumor effect, and 12 of 13 mice were found to be free of detectable hepatic tumors at the time of killing in this experiment ( $0.00 \pm 0.00$  g;  $n = 13$ ;  $P = 0.00003$  vs. untreated). This antitumor effect of chimeric SF-25 MAB was substantially inhibited by desulfated CGN (mice treated with chimeric SF-25 MAB and desulfated CGN,  $0.36 \pm 0.96$  g,  $n = 11$ ,  $P = 0.00005$  vs. mice treated with chimeric SF-25 MAB alone).

Nude mice were injected with 20  $\mu$ g of anti-asialo GM1 rabbit serum 5, 7, 9, and 12 days after tumor cell injection to deplete NK cells and treated with a single intravenous injection of chimeric SF-25 MAB (100  $\mu$ g per mouse 7 days after tumor cell injection). Results are shown in Figure 7B. The treatment of mice with anti-NK serum did not affect the growth of hepatic metastasis (untreated mice,  $1.31 \pm 1.31$  g,  $n = 15$ ; mice treated with anti-asialo GM1 rabbit serum,  $1.33 \pm 1.65$  g,  $n = 18$ ;  $P = 0.3745$ ) but significantly inhibited the antitumor effect of chimeric SF-25 MAB (mice treated with chimeric SF-25 MAB,  $0.14 \pm 0.25$  g,  $n = 14$ ; mice treated with chimeric SF-25 MAB and anti-asialo GM1 serum,  $1.09 \pm 1.33$  g,  $n = 8$ ;  $P = 0.0023$ ).

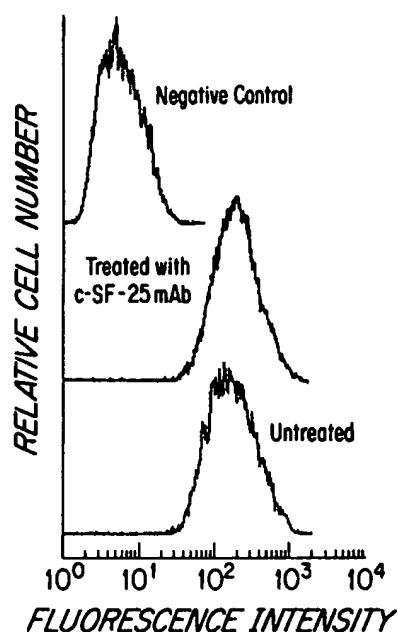
#### Expression of SF-25 Antigen in Liver Metastases After Chimeric SF-25 MAB Treatment

Although the outgrowth of hepatic micrometastases was inhibited by chimeric SF-25 MAB and the survival rate of mice was significantly improved, the in vivo purging of tumor cells was not perfect under our experimental conditions. Therefore, the expression of SF-25 antigen in the tumor cells from chimeric SF-25 MAB-treated mice was examined to investigate whether treatment selected for the growth of tumor cells that no longer bore the SF-25 antigen. However, as shown by flow cytometric analysis (Figure 8), the chimeric SF-25 MAB-treated tumor cells expressed SF-25 antigen at a similar level as those obtained from untreated mice. These experiments show that the outgrowth of tumor in treated mice was not the result of selection of SF-25-negative tumor cells.

#### Discussion

The properties of chimeric SF-25 MAB were determined in the present investigation, and it was found that (1) the chimeric SF-25 MAB construct retains binding specificity of the murine SF-25 MAB, (2) the antibody is not internalized or shed from the tumor cell surface





**Figure 8.** Expression of SF-25 antigen in LS 180 tumor after chimeric SF-25 MAb treatment. SF-25 antigen was examined by flow cytometric analysis using fluorescein isothiocyanate-labeled SF-25 MAb. The antigen was uniformly expressed at high density in the majority of the tumor cell populations (>99%) derived from both untreated and treated mice. Fluorescein isothiocyanate-labeled irrelevant MAb (B2T) served as a negative control.

on binding to the antigen, (3) chimeric SF-25 MAb elicits an ADCC by NK cells and macrophages but does not induce complement-mediated cytotoxicity, and (4) the observed high number of antibody binding sites ( $2.5 \times 10^5$  colon adenocarcinoma cell) and high association constant ( $2.4 \times 10^9$  mol/L) suggest that this MAb is delivered to the tumor cells in high density in vivo. All of these properties of chimeric SF-25 MAb are of interest and suggest that it may be effective as a potential immunotherapeutic reagent.<sup>26,27</sup>

An experimental animal model of hepatic metastasis of human colon cancer using athymic nude mice was used to study the in vivo antitumor effect of chimeric SF-25 MAb. Although this animal model is not a spontaneous metastatic model and does not represent all of the steps of the metastatic cascade as defined by Fidler,<sup>28</sup> it simulates metastatic growth of tumor cells in the liver after vascular spread. The subcutaneous xenograft models have often been used to test the antitumor effects of MAbs directed against tumor antigens.<sup>29,30</sup> However, the subcutaneous model of colon cancer rarely produces spontaneous hepatic metastasis. In addition, all mice with hepatic metastasis died from massive hepatic tumor involvement and hepatic dysfunction 5–7 weeks after tumor cell injection. Therefore, it is possible to objectively test the antitumor effects of a reagent such as

chimeric SF-25 MAb by examining the survival curves of the mice.

We have shown that the chimeric SF-25 MAb inhibited the outgrowth of the experimental metastatic tumors of human colon cancer established in the liver of nude mice. Especially noteworthy was the inhibition of tumor growth when treatment was started immediately after tumor cell injection. A complete inhibition of tumor growth was achieved and no tumor was detectable in the liver at the time of killing when control untreated animals are about to die from the large hepatic tumor burden. A substantial antitumor response was also produced against 5- and 7-day hepatic metastases by a single intravenous injection of chimeric SF-25 MAb. In these experiments, antitumor effects were achieved in a dose-dependent manner and inhibition of tumor growth was greater at higher doses than at lower doses of chimeric SF-25 MAb. Furthermore, the survival of the tumor-bearing animals was improved by chimeric SF-25 MAb administration as well. These results strongly support the concept that chimeric SF-25 MAb has in vivo antitumor properties. In this regard, possible antitumor mechanisms mediated by MAbs have been shown to include (1) induction of tumor cytotoxicity by effector cells such as NK cells and macrophages,<sup>31–33</sup> (2) activation of complement and induction of complement-mediated cytotoxicity,<sup>34</sup> (3) interference with cell growth or differentiation by binding growth factors or receptors on the surface of tumor cells,<sup>35,36</sup> and (4) induction of anti-idiotypic antibodies that subsequently act as novel vaccines against the tumor.<sup>37,38</sup>

It was found that F(ab')<sub>2</sub> fragment had little effect on the inhibition of hepatic tumor growth. This observation indicates that the Fc portion of the antibody was essential for a possible effector function of chimeric SF-25 MAb. Because chimeric SF-25 MAb was not capable of mediating complement-mediated cytotoxicity against LS 180 cells or the direct anti-tumor effect in vitro (unpublished data, January 1992), we further examined if cell-mediated mechanisms were involved as an explanation for the antitumor effects of chimeric SF-25 MAb. Because the immunologic defect of nude mouse is limited to T lymphocytes, NK cell and macrophage functions are intact.<sup>29,39,40</sup> Therefore, such effector cell populations may mediate tumor cell cytotoxicity in nude mice under our experimental conditions. It was found that the administration of anti-asialo GM1 antibody significantly inhibited the in vivo antitumor effects mediated through chimeric SF-25 MAb. Previous studies have shown that systemic injection of this antiserum decreases the number of hepatic asialo GM1-positive cells and NK cell functional activities in the liver.<sup>19,41,42</sup> Hepatic NK cell activ-

ities have been found to be important in inhibiting the growth of tumor metastasis.<sup>18,19,41-43</sup> Our data is consistent with the hypothesis that chimeric SF-25 MAb may have augmented this NK cell activity in an antibody-mediated fashion. Other experiments examined the role of the macrophages as effector cells in this experimental animal model system. Desulfated CGN was used to deplete macrophages in vivo because desulfated CGN has been previously shown to be effective for the depletion of resident macrophages in systemic organs as well as macrophages in peripheral blood.<sup>22</sup> The suppression of macrophages by desulfated CGN significantly inhibited the antitumor effect of chimeric SF-25 MAb. It is possible that the circulating macrophage populations and the liver resident macrophages (Kupffer's cells)<sup>44</sup> play an important role as effector cells in the inhibition of tumor growth mediated by chimeric SF-25 MAb.

Although chimeric SF-25 MAb elicits ADCC by murine effector cells in vitro like other chimeric MABs,<sup>45</sup> this finding does not imply that ADCC is the only important mechanism for the inhibition of metastatic tumor growth in vivo. It was possible that chimeric SF-25 MAB has augmented the tumoricidal properties of effector cells by unknown mechanisms other than ADCC, and these effects may not have been identified and tested in our in vitro assays. Furthermore, the data obtained in the nude mice model may as well underestimate as overestimate what may be achieved with this MAB in humans. Indeed, human effector cells are more cytotoxic to LS 180 human colon adenocarcinoma cells than these of nude mice in the ADCC assay (unpublished data, July 1990). Furthermore, antibodies injected into human subjects might induce a T cell-mediated immune response against colon tumors, and this effector mechanism is not testable in athymic nude mice. Thus, there are several limitations inherent in the nude mice xenograft model; nevertheless, this experimental animal model provides a functional system to investigate some of the in vivo antitumor properties of the chimeric SF-25 MAB.

Because chimeric SF-25 MAB was not able to completely irradiate hepatic micrometastasis, we determined if chimeric SF-25 MAB treatment may have selected SF-25 antigen-negative tumor cells that were resistant to chimeric SF-25 MAB treatment. The expression of SF-25 antigen was studied in the tumors derived from mice treated with chimeric SF-25 MAB. It was found that SF-25 antigen was indeed expressed in chimeric SF-25 MAB-treated tumor cells as shown by flow cytometric analysis. Therefore, the outgrowth of tumor in treated mice was not the result of selection of SF-25 antigen-negative tumors but may have resulted from insufficient dose or efficacy of chimeric SF-25 MAB for the tumor burden

and growth rate of the LS 180 cells. In addition, chimeric SF-25 MAB strikingly inhibited the outgrowth of tumor cells seeded into the portal vein and micrometastases established in the liver of nude mice and thus was a potent reagent for the prevention of the hepatic metastasis. The requirement of Fc fragment of the antibody as well as the depletion studies of NK cells and macrophages indicates that the antitumor effects of this antibody may be through cell-mediated mechanisms.

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